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Research Article

Prevalence of *Vibrio* Spp and Antibiogram of Isolates from Shrimp Rearing Ponds in Bangladesh

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ABSTRACT

Forty eight water samples were collected from 12 shrimp rearing ponds in Khulna Division, southern part of Bangladesh. Fifteen *Vibrio* spp were isolated and identified and subjected to antibiotic susceptibility testing against ten commercial antibiotic disks. 58% water samples were found positive for *Vibrio spp.* Among the isolates *V. fischeri* (40%) were the most prominent, followed by *V. vulnificus* (27%), *V. anguillarum* (26%), and *V. cincinnatiensis* (7%). Most of the *Vibrio* isolates were found to tolerate as much as 6% concentrations of NaCl and could not grow in culture medium without added salt. Antibiotic resistance pattern showed the highest resistance to ampicillin (100%), followed by amoxicillin (78%), nalidixic acid (40%), vancomycin (13.33%), neomycin (6.66%) and chloramphenicol (6.66%). All the Isolates were observed sensitive to gentamycin, erythromycin, ciprofloxacin and doxycyclin. Plasmid profiling of the isolates were conducted to observe if there any association of large plasmids with antibiotic resistance**.** Only 3 isolates were found to carry plasmid. Among them *Vibrio anguillarum*/P3 harbored four plasmids of 2.7, 3.0, 3.9 and 5.1 Kbp, *Vibrio anguillarum*/P1 harbored one plasmid of larger than 7.3 kbp and *Vibrio cincinnatiensis*/ P5H harbored one plasmid of larger than 30 kbp.

Keywords: Shrimp, *Vibrio,* biochemical characterization, antibiotic susceptibility, plasmid profile.

1. INTRODUCTION

Control of shrimp diseases is of a great concern in aquaculture because of the high risk of disease transmission, mortalities and economic losses [1]. Shrimp production, processing and export is an important source of foreign exchange earning of Bangladesh, taking place in 9000 farms covering an area of about 130 thousand hectares (12.7% of global area under shrimp culture) and producing an average output of 30 thousand tons annually (5% of the global production) [2]. Bacteria, viruses and protozoa have all been implicated as pathogens in shrimp culture, often causing significant economic losses. Vibriosis is one of the most prevalent enzootic diseases of fish all over the world that occurs among various fish species including marine, brackish and occasionally freshwater fishes [3]. Vibriosis is a term for several fish diseases causing serious problems for a wide range of wild and farmed species [4]. *Vibrios* are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, and mortalities up to 100% have been reported due to vibriosis. Shrimp pathogenic *Vibrios* are mainly *V.harveyi, V.fluvialis, V.parahaemolyticus, V. anguillarum, V.damsela* and *V.vulnificus* [5]. According to ICMSF and EU, the shrimp to be eaten must be free of *Vibrio*. *Vibrio* has been implicated in numerous outbreaks of sea food-borne gastroenteritis in the United States, which may have resulted from the consumption of raw or insufficiently heated minimally processed seafood or post processed contaminated sea food [6]. Vibriosis is endemic in many parts of Asia, and marked variation in the *in vitro* susceptibility to antibiotics of *Vibrio* has been observed, with emerging resistance to nalidixic acid, co-trimoxazole, furazolidone and streptomycin. The emergence of multidrug-resistant strains of *V. cholerae* O1 has been a matter of concern, as tetracycline is not recommended for use in children, and quinolones are also not advocated for use in children and pregnant women [7]**.** Plasmids are extra-chromosomal DNA found in bacteria and sometimes there is a correlation between possessions of the plasmid with antibiotic resistance [8-10]. Plasmid containing bacteria may transfer resistance to other bacteria by conjugation. For *vibrios* cases, the previous studies showed that this bacterial species contained plasmid [11-12]. In general, bacterial disease of shrimp has not been studied well in Bangladesh. Our present study was designed to examine the occurrence of *Vibrio* spp in the shrimp culturing ponds in Khulna Division, southern part of Bangladesh and to study their antibiotic resistance pattern and plasmid biology.

2. MATERIALS AND METHODS

2.1. Sampling site and collection of samples

Shrimp cultivation ponds are rapidly growing in southern part of Bangladesh and almost all shrimp industries have the aim to supply shrimp to the local and international market. For the present study, twelve shrimp culturing ponds were selected from three places of Khulna Division namely Moddokul (Kesobpur), Monirampur and Chinatola (Table 1). Sampling was done over a period of three months from December 2010 to February 2011 and total 48 samples were collected. Water sample was kept in ice box during transportation to the laboratory. Temperature and pH was recorded during sample collection.

2.2. Enrichment of sample

50 ml water sample was filtered by 0.45 µm cellulose nitrate filter (Sartorius AG, Germany) in aseptic condition. Then the filter paper was put into a conical flask containing 250 ml Alkaline Peptone water. The flask was covered with sterile aluminum foil and shaken 25 times clockwise and 25 times anti-clockwise to suspend the filtrates homogenously. This filter paper containing broth was incubated in a water bath at 42°C for 6-8 hrs

2.3. Isolation

3mm loopful of surface or pellicle growth from incubated alkaline peptone broth was streaked on to the surface of TCBS Agar (HiMedia, India). The plates were covered and inverted, and incubated at 35-37°C for 24±2 hrs. After incubation, typical or suspicious *Vibrio* colonies of yellow and green color were isolated, purified and preserved in nutrient agar (HiMedia, India) slant for final confirmation. All isolates were subjected to oxidase test and only oxidase positive cultures were selected for further biochemical study.

Table 1: Sampling site, average temperature and pH of the samples

Ponds	Location	Area of the Ponds	Number of Samples (N)	Average Temperature of the samples (^{0}C)	Average pH of the samples
1	Moddokul (Kesobpur)	1 acre	4	24^{0}C	9.7
2	Moddokul (Kesobpur)	1.3 acre	4	24^{0}C	9.8
3	Moddokul (Kesobpur)	100 acre	4	25^{0}C	9.6
4	Monirampur	2.7 acre	4	22^{0}C	9.2
5	Chinatola	1.7 acre	4	23^{0}C	9.8
6	Chinatola	2 acre	4	25^{0}C	9.8
7	Moddokul (Kesobpur)	1.5 acre	4	22^{0}C	9.6
8	Moddokul (Kesobpur)	1.8 acre	4	23^{0}C	9.2
9	Moddokul (Kesobpur)	1 acre	4	25^{0}C	9.8
10	Monirampur	2.7 acre	4	24^{0}C	9.8
11	Chinatola	2 acre	4	24^{0}C	9.7
12	Chinatola	4 acre	4	25^{0}C	9.8
			N=48		

2.4. Identification and biochemical characterization

Oxidase positive isolates were subjected to morphological and biochemical tests according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology, 9th edition [13]. The shape and Gram reaction were microscopically studied using 18 hour culture from agar slant. The biochemical tests used were Kliger's Iron (KIA) Agar test, Citrate utilization as sole source of carbon, Motility test, Indole test, urease test, Methyl red test, VP test, H_2S production in TSI agar, Lysine, ornithin and arginin decarboxylation, catalase test, growth in Nutrient Broth, fermentation of lactose, sucrose, glucose, starch, arabinose and mannose. Growth pattern in 5°C, 35°C and 45°C was also observed. All the media used for biochemical tests were modified with 2% NaCl.

2.5. Growth on salt medium

The isolates were subjected to salt tolerance test using NaCl (BDH). Nutrient broth (HiMedia, india) was modified with addition of 1%, 2%, 4%, 6%, 7%, 8% and 10% of NaCl. Isolates were inoculated in these modified broths and incubated at 35°C for 24 hours. Any degree of turbidity was considered

for positive growth. Salt tolerance of selected isolates was considered as a parameter for identification of the isolates

2.6. Antibiogram study

The antimicrobial susceptibility test was done by agar disc diffusion assay as described by NCCLS (2000), now known as the Clinical and Laboratory Standards Institute (CLSI) [14]. The antimicrobial agents used were Ampicillin 10µg, Amoxicillin 10µg, Vancomycin 30µg, Gentamycin 10µg, Erythromycin 15µg, Nalidixic Acid 30µg, Neomycin 30µg, Chloramphenicol 30µg, Ciprofloxacin 5µg and Doxycycline 30µg. Pure colonies of isolated *Vibrio* were emulsified in normal saline and turbidity was matched with 0.5 McFarland turbidity standards. Selected antibiotic discs were placed on Mueller Hinton Agar (HiMedia, India) (with 2% NaCl) plates seeded with bacteria. These plates were then incubated at 37°C for 24 hours. The organisms were observed for antibiotic sensitivity based on diameters of zones of inhibition on the petri dishes. Susceptible and resistant isolates were defined according to the criteria suggested by the CLSI.

2.7 Plasmid profiling of selected isolates

Plasmid extraction was done according to some modification of alkaline lysis method as described by Kado and Liu [15]. Isolates were grown overnight in LB broth (HiMedia, India) (with 2% NaCl) and cell pellet was collected by centrifugation in 13000 RPM for 5 minutes by a microcentrifuge (Sigma 2-16 k). Pellets were re-suspended in 20 µl of TE buffer (50mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed by adding 100 µl of lysis buffer (50 mM Tris, 3% SDS, pH 12.6) to each tube, gently mixed and incubated at 56° C for 30 minutes in a water bath. Then 100 µl of chilled Phenol: chloroform: iso-amyl alcohol (25:24:1) was added and shaken until the suspension was homogenous milky white. Then the mixture was centrifuged for 15 min at 13000 rpm. 40 µl from the upper aqueous phase was carefully removed to a clean eppendorf tube. This aqueous phase contains the plasmid. The isolated plasmid was electrophoresed on 0.8% agarose gel for 1 h at 90V, stained in ethidium bromide (0.5µg/ml) solution for 5 min and de-stained in distilled water for 1 min. The gel was then visualized on a UV trans-illuminator (Alpha Innotech). The molecular weight of plasmid DNA of the *Vibrio* isolates was determined using the graphical method relating the logarithm of the molecular weight of plasmids of *E. coli* V517. All reagents used for plasmid extraction were purchased from Invitrogen.

3. RESULTS

3.1. Identification of presumptive Vibrio spp in shrimp rearing ponds

3.1.1 Biochemical studyies

Initially 40 suspected colonies were selected from TCBS agar plate and finally 15 isolates were confirmed to belong to the genus *Vibrio* according to the limited description in Bergey's Manual of Determinative Bacteriology (8th edition, 1974). Results of biochemical tests are presented in Table 2. Among the 15 oxidase positive isolates, 11 isolates were yellow and 4 isolates were green colored on TCBS agar. All the 15 isolates were found indole negative, VP negative and were unable to use citrate as sole source of carbon. All isolates fermented mannose but not arabinose and could not break arginin and salicin. None of the isolates could grow without added NaCl in media. All 15 isolates were found to grow at 42°C and 9 isolates could grow at 5°C. On the basis of all other biochemical and morphological characteristics, isolates were found closely related to four species namely *V. anguillarum* (isolate P1, P2, P3, P43), *V. fischeri* (isolate P4, P4S, P5, P12S, P12Y, P12R), *V. vulnificus* (isolate P5S, P5E, P5F, P6G) and *V. cincinnatiensis* (isolate P5H). *V. anguillarum, V. fischeri and V. vulnificus* have been reported previously to be prevalent in shrimp culturing ponds [16].

3.1.2 Growth of the isolates on salt media

Marine *Vibrio* can tolerate considerable amount of salt [17]. In our present study, we tested all the 15 isolates to grow in nutrient broth containing NaCl up to 10%. All isolates failed to grow in media without NaCl. All isolates showed salt tolerance upto 6% NaCl. 53%, 27% and 13% isolates were found to grow at 7%, 8% and 10% concentration of NaCl respectively (Figure 1). Therefore all of our isolates may be considered as halotollerant. Increase of salt concentration cause the change of sensitivity toward antibiotics from the resistant to susceptible phenotype [18].

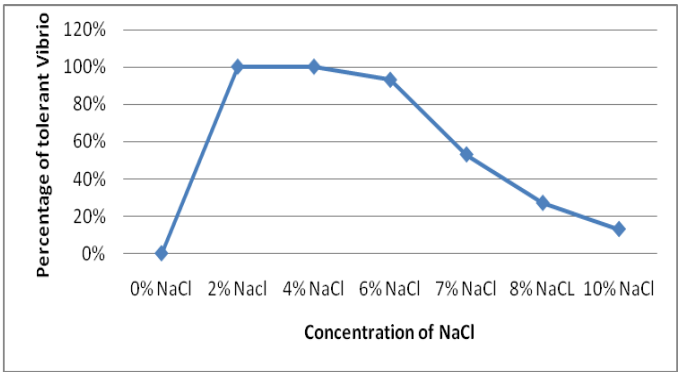

Figure 1: Effect of salt concentration on isolated Vibrio spp

Table 2: Biochemical characteristics of the Vibrio spp isolated from shrimp culturing ponds

Test parameters	Isolates Identified and their Biochemical Behavior														
	V.anguillarum /P1	*V.anguillarum*/P2	*V.anguillarum*/P3	*V. fischeri* /P4	*V. fischeri*/P4S	*V.anguillarum*/P43	*V. fischeri*/P5	*V. vulnificus*/P5S	*V. vulnificus* /P5E	*V. vulnificus* /P5F	*V.cincinnatiensis*/P5H	*V. vulnificus* /P6G	*V. fischeri* /P12S	*V. fischeri*/P12Y	*V. fischeri*/P12R
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	+	+	+	-	-	+	-	-	-	+	-	-	+	-
Mannitol	-	-	+	-	-	-	+	+	+	-	+	-	-	-	-
Starch	+	+	+	-	-	+	+	+	+	-	+	-	+	+	+
0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7% NaCl	+	+	-	-	+	+	-	-	-	+	+	+	-	-	+
10% NaCl	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-
5°C	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LIA	-	+	+	+	+	-	+	+	-	+	-	+	-	-	+
Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+

Note: + indicates positive reaction and – indicates negative reaction

3.2. Occurrence and distribution of the isolates

Among the ponds studied, *Vibrios* were found in seven ponds (58%) and other samples from pond 7 to 11 were free from *Vibrio* contamination (Table 3). Among the isolates, *V. fischeri* (40%) were the most prominent, followed by *V. vulnificus* (27%), *V. anguillarum* (26%), and *V. cincinnatiensis* (7%). The percentage of occurrence of these species is presented in figure 2.

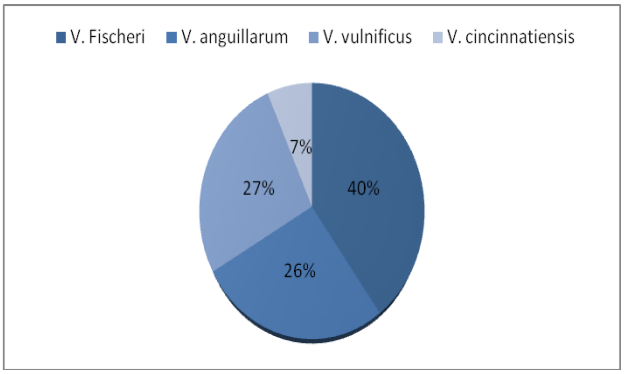

Figure2: Percentage of occurrence of Vibrio spp in the samples

Table 3: Occurrence and distribution of four species of Vibrio in different ponds studied

Ponds	Location	Number of Samples	*Vibrio* isolated
1	Moddokul (Kesobpur)	4	*V. anguillarum/P1*
2	Moddokul (Kesobpur)	4	*V. anguillarum/P2*
3	Moddokul (Kesobpur)	4	*V. anguillarum/P3*
4	Monirampur	4	*V. anguillarum/P43* *V. fischeri/P4* *V. fischeri/P4S*
5	Chinatola	4	*V. fischeri/P5* *V. vulnificus*/P5S *V. vulnificus*/P5E *V. vulnificus*/P5F *V. cincinnatiensis/P5H*
6	Chinatola	4	*V. vulnificus/P6*G
7	Moddokul (Kesobpur)	4	-
8	Moddokul (Kesobpur)	4	-
9	Moddokul (Kesobpur)	4	-
10	Monirampur	4	-
11	Chinatola	4	-
12	Chinatola	4	*V. fischeri*/P12S *V. fischeri*/P12Y *V. fischeri*/P12R

3.3. Antibiogram of the Vibrio spp isolates

It is reported that antibiotics are used with feed or some other means to inhibit shrimp pathogens in rearing ponds. With the increase of antibiotic use and due to many other environmental factors, marine *Vibrio spp* are gaining resistance against many antibiotics including nalidixic acid, co-trimoxazole, furazolidone and streptomycin [19]**.** As new antibiotics are developed and used, resistant strain may be developed because there is a tendency to assume that antibiotic resistance genes appeared only when antibiotics are used widely in medicine and fields industry [20]. Another studies have shown that streptomycin, rifampicin, kanamycin, tetracycline, polymyxin B were active against *Vibrio* spp [21-22]. In our present investigation all of 15 isolates were subjected to antibiotic susceptibility testing against 10 commercial antibiotic disks. The result was interpreted according to CLSI guideline and presented in table 4 and figure 3. Among the drugs used, isolates showed resistance to 6 drugs. The highest resistance was found to ampicillin (100%) followed by amoxicillin (78%), Nalidixic acid (40%), Vancomycin (13.33%), Neomycin (6.66%) and chloramphenicol (6.66%). All Isolates showed sensitivity to gentamycin, erythromycin, ciprofloxacin and doxycyclin. Vancomycin, neomycin and chloramphenicol sensitivity was found 86.66%. Ampicillin resistance in our study is very similar to other studies that have been reported, which was ranging from 44.4% to 98% [23-24].

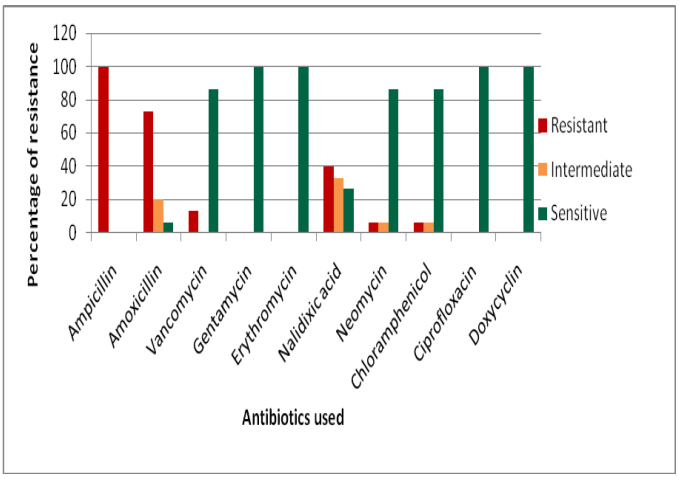

Figure 3: Percentage of resistant, intermediate and sensitive Vibrio isolates

3.4. Plasmid profile of the Vibrio spp

Bacterial antibiotic resistance patterns sometimes associated with the presence of large plasmids. Generally, plasmids which can be transconjugated usually possess a high

Table 4: Antibiotic susceptibility pattern of 15 Vibrio spp isolates

Isolated *Vibrio spp*	**Antibiotics**									
	Ampicillin 10µg	Amoxicillin 10µg	Vancomycin 30µg	Gentamycin 10µg	Erythromycin 15µg	Nalidixc Acid 30µg	Neomycin 30µg	Chloramphenicol 30µg	Ciprofloxacin 5µg	Doxycyclin 30µg
Vibrio anguillarum /P1	R	S	S	S	S	S	S	S	S	S
Vibrio anguillarum /P2	R	I	S	S	S	R	R	I	S	S
Vibrio anguillarum /P3	R	R	R	S	S	I	I	S	S	S
Vibrio fischeri /P4	R	R	S	S	S	R	S	R	S	S
Vibrio fischeri/P4S	R	R	S	S	S	R	S	S	S	S
Vibrio anguillarum/ P43	R	R	S	S	S	I	S	S	S	S
Vibrio fischeri/P5	R	R	S	S	S	R	S	S	S	S
Vibrio vulnificus/ P5S	R	R	S	S	S	I	S	S	S	S
Vibrio vulnificus /P5E	R	R	S	S	S	R	S	S	S	S
Vibrio vulnificus /P5F	R	R	R	S	S	I	S	S	S	S
Vibrio cincinnatiensis/ P5H	R	R	S	S	S	I	S	S	S	S
Vibrio vulnificus /P6G	R	I	S	S	S	S	S	S	S	S
Vibrio fischeri /P12S	R	R	S	S	S	S	S	S	S	S
Vibrio fischeri /P12Y	R	I	S	S	S	R	R	S	S	S
Vibrio fischeri /P12R	R	R	S	S	S	S	S	S	S	S

Note: R-Resistant, I-Intermediate, S-Sensitive

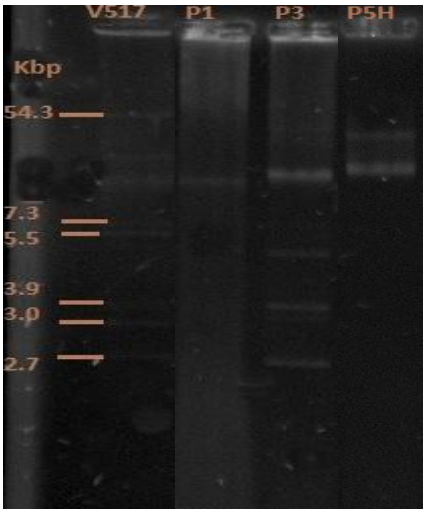

Figure 4: Plasmid profile of the Vibrio isolates on Agarose gel. 1st lane shows the marker bands of V517, 2nd lane shows one plasmid of >7.3 kbp of isolate P1, 3rd lane shows four plasmids of 2.7, 3.0, 3.9 and 5.5 kbp of isolate P3 and 4th lane shows >30 kbp plasmid of isolate P5H

molecular weight so the presence of plasmids may increase their capacity to threaten human consumers since foodborne strains carrying resistant genes qualified them as potential human pathogens. However, for others isolate that antibiotic gene may be associated in chromosomal DNA. In our present study all 15 *Vibrio* isolates were investigated for the presence of plasmid DNA. Only 3 isolates were found to carry plasmid (Figure 4). Among them *Vibrio anguillarum* /P3 was found to carry four plasmids of 2.7, 3.0, 3.9 and 5.1 Kbp and this isolate showed resistance against 3 antibiotics. *Vibrio anguillarum* /P1 harbored one plasmid of larger than 7.3 kbp and *Vibrio cincinnatiensis*/ P5H harboured one plasmid of larger than 30 kbp. The isolate *Vibrio anguillarum* /P1 were resistant to one antibiotic and *Vibrio cincinnatiensis*/ P5H was resistant against two antibiotics. No correlation of carrying plasmid and drug resistance was observed.

4. CONCLUSION

From our present study it may be concluded that shrimp culturing ponds in southern part of Bangladesh are getting contaminated with drug resistant *Vibrio spp.* Therefore, it is very important to pay attention to good rearing practice, hygiene, use of antibiotic supplemented feed and pollution in the shrimp rearing ponds. Furthermore, investigation and research are required to find out the root cause of *Vibrio* prevalence in shrimp rearing ponds in Khulna, Bangladesh.

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